REMARKS

Status of the Claims

Claims 1-4, 6-9, and 13-24 are pending in the present application. Claims 5, 10, and 12 are presently canceled. Claim 11 was previously canceled. Claims 14, 17-19, and 22-24 are withdrawn as directed to a non-elected invention. Claims 1, 3, 4, 6, 15, and 16 are amended. Support for the amendments is found throughout the application as originally filed including on page 21, page 18, and in previously pending claims 5, 10, and 12. No new matter is entered by way of this amendment. Reconsideration is respectfully requested.

Issues Under 35 U.S.C. § 112, first paragraph

Written Description

Claims 1-10, 12, 13, 15, 16, 20, and 21 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description paragraph, *see* Office Action, pages 2-4, item 4. Applicants respectfully traverse.

Specifically, the Examiner states that there is insufficient written description to demonstrate support for the genus of fibronectin fragments or polypeptides having a substitution, deletion, insertion, or addition to one or more fibronectin fragments that comprise a cell adhesion activity and/or a heparin binding activity. The Examiner does not believe that an ordinary artisan at the time of the invention could have envisioned the members of the described genus of fibronectin fragments.

In an effort to expedite prosecution the claims are amended to cancel the subject matter that the Examiner believes is not supported by the present application. In addition, independent claim 1 is amended to specify a recombinant fibronectin fragment, wherein the recombinant fibronectin fragment is a polypeptide comprising at least any one of the amino acid sequences shown in SEQ ID NOs: 1 to 20 and 25 of the sequence listing. Applicants submit that an ordinary artisan is able to envision the genus of fibronectin fragments encompassed by independent claim 1 and the dependent claims incorporating this genus. In view of the foregoing, the rejection is overcome and withdrawal of the rejection is respectfully requested.

Enablement

Fibronectin Fragments

Claims 1-10, 12, 13, 15, 16, 20, and 21 are also rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement, *see* Office Action, pages 4-6, item 5. Applicants respectfully traverse.

Specifically, the Examiner states that the originally filed application fails to provide sufficient examples, which demonstrate that the broad genus of fibronectin fragments described in the present claims may be used to increase the number of cytotoxic cells. In particular, the Examiner states that the specification describes an N-terminal Type 1 repeat of fibronectin comprising a heparin binding domain. However, according to the Examiner, the instant specification provides insufficient guidance as to what sequences from this region may be used to increase the number of cytotoxic cells.

In an effort to expedite prosecution, claim 1 is amended, as described above, to specify that the recombinant fibronectin fragments comprise SEQ ID NOs: 1 to 20 and 25. These sequences are described in the present application as having cell binding and/or heparin binding activity, *see*, for example, pages 13-15. The specification further teaches that sequences having these activities may be used to increase cytotoxicity, *see*, for example, pages 8-9 of the originally filed application. Accordingly, Applicants believe the rejection is overcome and respectfully request withdrawal thereof.

Precursor Cells

The Examiner further alleges that only peripheral blood mononuclear cells are suitable precursor cells for differentiating cytotoxic lymphocytes, *see* Office Action, item 5, page 6. According to the Examiner, the additional cell types encompassed by the present claims (*e.g.*, umbilical cord blood mononuclear cells) may not predictably be used to form cytotoxic lymphocytes.

In particular, the Examiner indicates that the use of umbilical cord blood mononuclear cells for differentiation into a cytolytic lymphocyte population with fibronectin and IL-2 is unpredictable. To support this contention, the Examiner cites Lucivero *et al.*, *Int. J. Clin. Lab. Res.*, 1996, 26:255-261, ("Lucivero"), which allegedly teaches that umbilical cord blood lymphocytes are different in phenotype and function from lymphocytes of normal adults and

display a functionally immature phenotype. The Examiner further asserts that Lucivero teaches that anti-CD3 stimulants fail to induce proliferation of cord blood lymphocytes. Accordingly, the Examiner asserts that differentiation of cord blood lymphocytes into a population of cells comprising enhanced cytolytic activity would be highly unpredictable.

An ordinary artisan would have recognized from the present specification and the art known at the time of the invention that umbilical cord blood mononuclear cells could have been used with the claimed methods

Independent claim 1 is amended to specify that the precursor cells are peripheral mononuclear cells and umbilical cord blood mononuclear cells.

As noted above, the Examiner indicates that an ordinary artisan recognizes that peripheral mononuclear cells may be used with the claimed methods. However, the Examiner believes that an ordinary artisan would not have recognized that umbilical cord blood mononuclear cells could also have been predictably used. In contrast to the Examiner's assertions, Applicants submit that an ordinary artisan would have understood from the present application and the art known at the time of the invention that umbilical cord blood mononuclear cells would have been a suitable precursor population for differentiation into cytotoxic lymphocytes.

In support thereof, Applicants submit herewith, D.L. Nelson *et al.*, "The Production of Soluble and Cellular Interleukin-2 Receptors by Cord Blood Mononuclear Cells following *In Vitro* Activation," *Pediatric Research*, 1986, vol. 20, no. 2, pp. 136-139, ("Nelson"), which was published prior to the filing date of the instant application.

Nelson teaches that peripheral mononuclear cells *or* umbilical cord blood mononuclear cells may be cultured in a medium containing OKT3, which is an anti-CD3 antibody, to obtain activated cells. Nelson further teaches that IL-2R is increased in activated umbilical cord blood mononuclear cells. Applicants submit that, at the time of the invention, an ordinary artisan would have recognized that IL-2R, which is expressed on the surface of an activated T cell, is a marker for activation. That is, upon expression of IL-2R, cytokine production, cytotoxic activity, proliferation or the like is activated, *see* page 4, lines 18 to 20 and page 25, lines 12 to 15 in the originally filed application. *See also* Example 25, page 95, of the originally filed application. Accordingly, an ordinary artisan would have understood at the time of the invention that IL-2R expression is an indicator of cytotoxic lymphocyte formation. Therefore, an ordinary artisan

would have recognized from the state of art and Nelson that peripheral mononuclear cells *or* umbilical cord blood mononuclear cells may be cultured, using the same medium, to obtain cytotoxic lymphocytes.

Applicants further submit that one of ordinary skill would have known, as a matter of fact, that both peripheral mononuclear cells and umbilical cord blood mononuclear cells are monocytes and that these cells have similar characteristics and properties. Accordingly, one of ordinary skill in the art would have readily understood from Nelson and the present application that the production of cytotoxic lymphocytes could have been accomplished by culturing peripheral mononuclear cells *or* umbilical cord blood mononuclear cells in the same medium.

The Examiner further asserts that anti-CD3 antibody stimulation is not essential to the proliferation and induction of umbilical cord blood mononuclear cells, solely on the basis of Luciverto. However, although Luciverto allegedly teaches that anti-CD3 stimulants fail to induce proliferation of cord blood lymphocytes, an ordinary artisan would have recognized from other art known at the time of the invention, such as Nelson, that cytotoxic lymphocytes may be produced from umbilical cord blood mononuclear cells by anti-CD3 antibody stimulation. Accordingly, Applicants submit that the Examiner supports his contentions using technical information in an unreasonably narrow manner.

In view of the foregoing, Applicants submit that the present application adequately supports the amended claims. Withdrawal of the rejection is respectfully requested.

Issues under 35 U.S.C. § 103(a)

Darfler, Ochoa, Cardarelli, Taguchi, and, optionally, Chen

Claims 1-10, 12, 13, 15, and 16 are rejected under 35 U.S.C. § 103(a) as allegedly obvious over PCT Publication No. 88/02774 to Darfler, ("Darfler"), in view of Ochoa *et al.*, *Cancer Res.*, 1989, 49:963-968, ("Ochoa"), Cardarelli *et al.*, *Cell Immunol.*, 1991, 135:105-117, ("Cardarelli"), and U.S. Patent No. 5,198,423 to Taguchi *et al.*, ("Taguchi"), *see* Office Action, pages 6-8, item 7.

Claims 20 and 21 are rejected under 35 U.S.C. § 103(a) as allegedly obvious over Darfler, Ochoa, Cardarelli, Taguchi, and U.S. Patent No. 5,198,423 to Chen *et al.*, ("Chen"), *see* Office Action, pages 8-9, item 8. The above described rejections are respectfully traversed.

Basis for the Rejection

According to the Examiner, Darfler describes incubating Peripheral Blood Mononuclear cells, ("PBMCs"), in serum-free media with IL-2 to obtain lymphokine activated killer cells, ("LAK"). The Examiner admits that Darfler does not describe using fibronectin to obtain differentiated cells. However, the Examiner believes that Ochoa and Cardarelli remedy this deficiency. The Examiner states that Ochoa describes adding anti-CD3 antibodies to boost proliferation while maintaining LAK activity. The Examiner further states that Cardarelli teaches adding immobilized fibronectin and IL-2 to PBMC cultures stimulated with anti-CD3 antibody in the presence of serum-free media to enhance proliferation and IL-2R expression of T lymphocytes. Taguchi is cited for describing a biologically active recombinant fibronectin fragment comprising SEQ ID NO: 13. Chen is cited for describing transduction of a foreign gene into a cell.

Legal Standard for Obviousness

The burden is on the Examiner to make a *prima facie* case of obviousness, which requires an objective analysis as set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966). In *KSR International v. Teleflex Inc.*, 82 USPQ2d 1385 (2007), the Court affirmed that this analysis includes the following factual inquires: (1) determining the scope and content of the prior art; (2) ascertaining the differences between the claimed invention and the prior art; and (3) resolving the level of ordinary skill in the pertinent art. The Examination Guidelines for Determining Obviousness Under 35 U.S.C. § 103 in view of the Supreme Court Decision in *KSR International Co. v. Teleflex Inc.* state that, having undertaken the factual inquires of *Graham*, a rejection under 35 U.S.C. § 103 may be supported by one or more of the following rationales: (1) combining prior art elements according to known methods to yield predictable results; (2) simple substitution of one known element for another to obtain predictable results; (3) use of a known technique to improve similar devices in the same way; (4) applying a known technique to a known device ready for improvement to yield predictable results; choosing from a finite number of identified, predictable solutions, with a reasonable expectation of success; (5) variations that would have been predictable to one of ordinary skill the art; and (6) some teaching, suggestion, or motivation in the prior art that would have led one of ordinary skill to modify the prior art reference or to combine the prior art reference teachings to arrive at the claimed invention. 72

Fed. Reg. 57526, at 57529 (October 10, 2007). Each of the above-noted rationales requires predictability in the art and/or a reasonable expectation of success, and the Examiner must consider objective evidence, which rebuts such predictability and reasonable expectation of success. This objective evidence or secondary considerations may include unexpected results and/or failure of others (*e.g.*, evidence teaching away from the currently claimed invention), evidence of commercial success, and long-felt but unsolved needs, as found in the specification as-filed or other source. *Id.* When considering obviousness of a combination of known elements, the operative question is "whether the improvement is more than the predictable use of prior art elements according to their established functions." *KSR* at 1396.

The Instant Invention

As amended, independent claim 1 is directed to a method for preparing a cytotoxic lymphocyte which method comprises the step of carrying out at least one step selected from the group consisting of induction from peripheral mononuclear cells or umbilical cord blood mononuclear cells which can be formed into the cytotoxic lymphocyte, maintenance of a cytotoxic lymphocyte and expansion of a cytotoxic lymphocyte, comprising culturing the peripheral mononuclear cells or umbilical cord blood mononuclear cells which have an ability of differentiating into the lymphocyte with a medium containing serum and plasma at a total concentration of 0% by volume or more and less than 5% by volume, in the presence of a recombinant fibronectin fragment, wherein the recombinant fibronectin fragment is a polypeptide comprising at least any one of the amino acid sequences shown in SEQ ID NOs: 1 to 20 and 25 of the sequence listing wherein said fibronectin fragment comprises a cell adhesion activity and/or a heparin binding activity, and wherein a cytotoxic activity is enhanced or a high cytotoxic activity is maintained as compared to a cytotoxic activity of a cytotoxic lymphocyte prepared in the absence of the recombinant fibronectin fragment.

An ordinary artisan could not have predictably achieved the claimed invention

Applicants submit that an ordinary artisan at the time of the invention could not have reasonably predicted from the cited references that incubating a recombinant fibronectin fragment with peripheral mononuclear cells or umbilical cord blood mononuclear cells in substantially

serum-free medium, as described in the instant claims, would result in enhanced cytotoxicity or the maintenance of a high cytotoxic activity.

Cardarelli describes a native fibronectin. In contrast, the claimed method employs a recombinant fibronectin fragment. Moreover, Cardarelli does not teach that fibronectin enhances cytotoxicity, but only teaches that fibronectin enhances proliferation, *see* Cardarelli, which states that T cell proliferation is measured by determining the amount of ^{3}H-thymidine uptake upon DNA synthesis of cells, *see* pages 107-108 of Cardarelli. In addition, Darfler's method describes using a compound for activating kinase C in order to obtain a serum-free medium, which has the equivalent function of a serum containing medium. However, kinase C activating compounds are not utilized in the claimed method. In view of the foregoing, an ordinary artisan could not have reasonably predicted that combining the serum-free medium of Darfler with a recombinant fibronectin fragment in the presence of peripheral mononuclear cells or umbilical cord blood mononuclear cells could have resulted in enhanced cytotoxicity or maintaining a high level of cytotoxicity.

Applicants further submit that neither Taguchi, Ochoa, or Chen remedy this uncertainty. Therefore, in view of the foregoing, an ordinary artisan could not have reasonably predicted from the combination of cited references that the incubation of peripheral mononuclear cells or umbilical cord blood mononuclear cells in substantially serum-free medium, in the presence of a recombinant fibronectin fragment, could have enhanced or maintained a high level of cytotoxicity upon induction of the described precursor cells.

Based upon the above, the claims are not rendered obvious by the cited references. Withdrawal of the rejection is respectfully requested.

Sagawa, Johnson, Darfler, Freshney and, optionally, Chen

Claims 1-8, 10, 12, 13, 15, and 16 are also rejected under 35 U.S.C. § 103(a) as allegedly being obvious in view of PCT Publication No. WO 02/14481 to Sagawa *et al.*, ("Sagawa"), as evidenced by the teachings of U.S. Publication No. 2005/0042208, which is the national stage application of Sagawa, in view of Johnson *et al.*, *J. Immunol.*, 1992, 148:63-71, ("Johnson"), Darfler, and Freshney ed., IRL Press, Animal Cell Culture, A Practical Approach, 1986, pages 26-41, ("Freshney"), *see* Office Action, pages 9-10, item 9.

Claims 20-21 are rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Sagawa, as evidenced by U.S. Publication No, 2005/0042208, Johnson, Darfler, Fresheny, and Chen, *see* Office Action, pages 10-11, item 10. The above-described rejections are respectfully traversed.

According to the Examiner, Sagawa describes inducing naïve T cells to cytotoxic lymphocytes using a fibronectin fragment. The Examiner acknowledges that Sagawa does not particularly describe using a serum-free medium. However, the Examiner states that Darfler remedies this deficiency. The Examiner believes that it would have been obvious to modify the method of Sagawa to include serum-free medium since Darfler teaches the advantages of such media. Freshney is cited for teaching that serum-free medium was commonly used at the time of the invention in mammalian cell culture. Johnson is cited for teaching that isolated T cells, grown in the presence of serum-free medium, anti-CD3 antibody, and IL-2 results in increased proliferation. Chen is cited for teaching the transduction of foreign genes into T cells.

Initially, Applicants note that Sagawa does not correspond to U.S. Publication No. 2005/0042208 as described in the present Office Action, but to U.S. Publication No. 2004/0115809, *see* Information Disclosure Statement, filed on June 7, 2010. U.S. Publication No. 2005/0042208 corresponds to PCT Publication No. 03/016511, which was published on February 27, 2003.

The Examiner has failed to establish a prima facie case of obviousness

Claims 1-8, 10, 12, 13, 15, and 16

The claimed invention allows for the induction of cytotoxic lymphocytes, which can not be induced under serum-free or low-serum medium conditions, by using a recombinant fibronectin fragment in combination with such media. *See also* Example 25, page 95 of the originally filed application. Applicants submit that an ordinary artisan would not have the proper rationale or have been motivated to combine the cited references as described in the instant Office Action since an ordinary artisan could not have reasonably expected that such combination could have predictably resulted in the induction of cytotoxic lymphoctyes.

Sagawa teaches that cytotoxic lymphocytes are induced in the presence of a fibronectin fragment in a serum-containing medium. Sagawa's method, however, also employs an antigen presenting cell as an essential element. Applicants note that the claimed method does not require

an antigen presenting cell. Accordingly, the claimed method is completely different from Sagawa. Applicants further note that Darfler teaches that LAK cells are induced by adding a compound for activating kinase C in a serum-free medium, which is also not necessary to the instantly claimed method.

Applicants submit that one of ordinary skill in the art would not have the proper rationale or have been motivated to combine Sagawa with Darfler. Due to the different essential elements described in Sagawa and Darfler, an ordinary artisan could not have been reasonably certain that merely substituting the serum-containing medium of Sagawa with the serum free medium of Darfler would have predictably resulted in cytotoxic lymphocyte induction from the naïve T cells described in Sagawa.

Johnson and Freshney fail to remedy this unpredictability. Johnson describes isolated T cells, not the naïve T cells described in Sagawa. Freshney merely describes that serum-free medium is used in the general culture of animal cells, and never suggests the induction of cytotoxic lymphocytes using a serum-free medium. Accordingly, an ordinary artisan would not have the proper rationale or have been motivated to combine the methods of Johnson and Freshney with those of Sagawa.

Applicants further note that the present claims describe peripheral mononuclear cells or umbilical cord blood mononuclear cells. In contrast, Johnson describes isolated T cells. Accordingly, the starting material described in the claimed invention differs from that of Johnson.

Based upon the foregoing an ordinary artisan would not have the proper rationale or have been motivated to combine the cited references and such combination would have resulted in a method that is completely different from the claimed invention. Accordingly, claims 1-8, 10, 12, 13, 15, and 16 are not rendered obvious by the cited references. Withdrawal of the rejection is respectfully requested.

Claims 20 and 21

Applicants further submit that dependent claims 20 and 21 are not rendered obvious by Sagawa, Johnson, Darfler, and Fresheny. Further, Chen fails to remedy the deficiencies of these references. Chen is merely cited for describing the transduction of foreign genes into T cells.

Accordingly, Applicants submit that claims 20 and 21 are also not rendered obvious in view of the cited art. In view of the above, withdrawal of the rejection is respectfully requested.

Non-Statutory Obviousness Type Double Patenting

Claims 1-8, 10, 12, 13, 15, 16, 20, and 21 are provisionally rejected on the ground of non-statutory double patenting as allegedly obvious over claims 1-3, 5-7, 10, 12, 28, 29, 31-35, and 37-39 of co-pending U.S. Application No. 10/509,055 in view of Johnson, Darfler, and Fresheny, *see* Office Action, pages 11-12, item 12. Applicants respectfully traverse.

According to the Examiner, the cited claims fail to describe medium containing less than 5% serum. However, the Examiner asserts that Johnson, Darfler and Fresheny remedy this deficiency.

As noted above, Applicants submit that an ordinary artisan could not have reasonably expected that the cytotoxicity of differentiated peripheral mononuclear cells or umbilical cord blood mononuclear cells could have been enhanced or a high cytotoxicity activity maintained by incubating the described precursor cells with a combination of recombinant fibronectin fragment and serum free medium in view of Johnson, Darfler and Freshney. *See also* Example 25, page 95 of the originally filed application, as noted above, which demonstrates the results of the claimed invention. Accordingly, Applicants believe the rejection is overcome and respectfully request withdrawal.

CONCLUSION

In view of the above amendment and remarks, Applicants believe the instant application is in condition for allowance. Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact L. Parker, Ph.D., Registration No. 46,046, at the telephone number of the undersigned below to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Director is hereby authorized in this, concurrent, and future replies to charge any fees required during the pendency of the above-identified application or credit any overpayment to Deposit Account No. 02-2448.

Dated: OCT 07 2010

Respectfully submitted,

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Attachments

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The Production of Soluble and Cellular Interleukin-2 Receptors by Cord Blood Mononuclear Cells following *In Vitro* Activation

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ABSTRACT. Cord blood mononuclear cells (CBMC) were investigated for their capacity to generate both cellular and soluble, supernatant interleukin-2 receptors (IL-2R) following cellular activation *in vitro*. Soluble IL-2R were measured in cell-free supernatants and in detergent-solubilized cell extracts with a "sandwich" enzyme-linked immunosorbent assay. CBMC and adult peripheral blood mononuclear cells were activated with phytohemagglutinin or the murine monoclonal antibody OKT3. CBMC and adult peripheral blood mononuclear cells generated cellular and soluble IL-2R in response to both activators. Peak values for supernatant IL-2R were observed on day 7, while peak values of cell-associated IL-2R occurred on day 5, followed by a decline on day 7. With the exception of supernatant IL-2R production induced by OKT3 stimulation, CBMC produced IL-2R in amounts comparable to adult mononuclear cells. Cord blood plasma also contained amounts of IL-2R comparable to that found in adult sera/plasma. Thus, CBMC appear largely immunocompetent with regard to the expression of IL-2R. (*Pediatr Res* 20: 136–139, 1986)

Abbreviations

CBMC, cord blood mononuclear cells
IL-2, interleukin-2
IL-2R, interleukin-2 receptor(s)
FCS, fetal calf serum
PBMC, peripheral blood mononuclear cells
PBS, phosphate buffered saline
PHA, phytohemagglutinin

The immune responsiveness of neonatal T cells appears relatively well developed. Neonatal T cells proliferate in response to polyclonal activators such as phytohemagglutinin (PHA) (1) and also to certain antigens such as cell-associated alloantigens (2). Neonatal T cells are also capable of providing both help and suppression for B-cell immunoglobulin responses induced by polyclonal activators (3, 4) and also help and suppression for specific antibody responses by allogeneic B cells induced by antigens *in vitro* (5). Neonatal T cells are capable of inhibiting proliferative responses of adult T cells to certain stimuli—presumably through both cellular and soluble mechanisms (6, 7). Recently, the role of the lymphokine IL-2 in immune responses has been under intense investigation (8). IL-2, which supports the proliferation of activated T cells and B cells (9), is secreted in normal amounts by PHA-activated neonatal T cells (10). While the normal proliferative responses noted above suggest that IL-2 expression might be normal in neonatal T cells, some controversy exists as to the presence of IL-2-independent mechanisms of T-cell proliferation (11) which might be employed by neonatal T cells. Therefore, we undertook an analysis of the expression of IL-2R by activated neonatal mononuclear cells *in vitro* using a newly developed enzyme-linked immunosorbent assay to quantitatively measure both cellular and released IL-2R.

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MATERIALS AND METHODS

Preparation of cord blood and adult peripheral blood mononuclear cells. CBMC and PBMC were prepared as previously described in detail by centrifugation on Ficoll-Hypaque gradients (5). Cord blood plasma and adult plasma were prepared by centrifugation of heparinized whole blood.

Antibodies. The murine monoclonal anti-IL-2R antibody termed anti-Tac (12) was the generous gift of Dr. T. A. Waldmann, NIH. The murine monoclonal anti-IL-2R, 7G7B6, which binds to the IL-2R at an epitope distinct from anti-Tac and IL-2, was produced and modified with fluorescein isothiocyanate as described (13).

Cell cultures and stimulants. Cultures of CBMC and adult PBMC containing 1×10^6 mononuclear cells in 2 ml of RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin 100 U/ml, and streptomycin 100 μg/ml were established in individual wells of 24-well plates (Costar). Triplicate cultures were incubated for various times in a humidified atmosphere of 5% CO_2 in air at 37° C. Cultures were either unstimulated (media alone), or they contained PHA (GIBCO) at a final dilution of 1/200 or the murine monoclonal antibody OKT3 (Ortho) at a final concentration of 10 μg/ml. Cultures were harvested by centrifugation at 550 × *g* for 10 min and the supernatants removed. The remaining cells were washed once in Dulbecco's phosphate buffered saline (PBS) containing 25 mM Tris pH 7.4. The cell pellet was solubilized in 0.5 ml of PBS containing 25 mM Tris pH 7.4 containing 1% Triton X-100, followed by centrifugation in a microfuge (EPPENDORF) for 5 min and recovery of the supernatant. Culture supernatants and detergent-solubilized cell extracts were stored at −20° C until assayed for IL-2R.

Enzyme-linked immunosorbent assay for the measurement of IL-2R. The enzyme-linked immunosorbent assay for soluble IL-2R was performed as described in detail (14). In brief, alternate columns of the inner 60 wells of microtiter plates were coated with 150 μl of the monoclonal anti-IL-2R antibody, anti-Tac, suspended in carbonate buffer pH 9.6 at a concentration of 1

μg/ml or buffer alone. Following overnight incubation, the plates were washed and 100 μl of sample was added to coated and control wells. Following a 2-h incubation at room temperature, the plates were washed and all wells received 100 μl of a 1:4000 dilution of fluorescein isothiocyanate-modified 7G7B6 in PBS containing Tween and 1% FCS (PBS/Tween/FCS). After a 2-h incubation, the plates were washed and 100 μl of a 1:1000 dilution of alkaline phosphatase-conjugated rabbit anti-fluorescein isothiocyanate in PBS/Tween/FCS added to all wells. After an additional 1-h incubation, p-nitrophenyl phosphate (1 μg/ml, Sigma) in diethanolamine buffer pH 9.8 was added and the absorbance of the control wells was subtracted from the experimental wells; this absorbance value was compared to absorbances determined for a standard curve generated by the addition of varying amounts of IL-2R as previously described (15). The IL-2R standard was the cell-free supernatant of an *in vitro* passaged T-cell line which was assigned a level of 1000 IL-2R/ml.

Calculations. Geometric means and SEM were calculated for the responses of groups of donors, and those values less than 62.5 were assigned a value of 62.5 for the purposes of calculations. Different groups were compared using Student's *t* test for nonpaired values.

RESULTS

Cellular and supernatant IL-2R generation by CBMC and PBMC with various stimuli. Previous studies of IL-2R generation by activated adult PBMC revealed that cellular IL-2R expression peaked on days 3–5 of culture while soluble supernatant IL-2R levels were maximal on day 7 of culture (14). Therefore, we first examined the cellular IL-2R production of stimulated CBMC and adult PBMC on day 5 of culture. As can be seen in Table 1, when CBMC and adult PBMC were stimulated with PHA or the murine monoclonal antibody OKT3, the cells of all donors expressed IL-2R on day 5 of culture. Cells from some donors made IL-2R when stimulated with culture medium alone. There were differences among both adult and neonatal individuals in terms of the capacity to generate IL-2R, and "high responders" tended to make larger quantities of cellular IL-2R with all stimuli, including media alone. With all of the stimuli tested, there were no significant differences between adult and neonatal mononuclear cells for the generation of cell-associated IL-2R. When the supernatants of these same 5-day cultures were examined for soluble IL-2R, the mean (×/÷ SEM) values were as follows: CBMC–media 63.5 (1.0), PHA 1275 (1.5), OKT3 271 (1.3); PBMC–media 58.9 (1.1), PHA 923 (1.4), and OKT3 725 (1.2). Again, for each stimulus tested, there was no statistically significant difference ($p < 0.01$) between CBMC and adult PBMC. We next examined the levels of soluble IL-2R in the supernatant of day 7 cultures from these same individuals. As can be seen in Table 2, activated CBMC and PBMC all produced measurable IL-2R. Those individuals who had generated the most cell-associated IL-2R on day 5 also produced the most supernatant IL-2R on day 7. With the exception of the response to OKT3, there were no significant differences between CBMC and PBMC with regard to the generation of soluble IL-2R on day 7 following activation. When the cells from four of the CBMC and four of the PBMC were studied for cell-associated IL-2R on day 7, the mean (×/÷ SEM) values were as follows: CBMC–media 62.5 (1.0), PHA 773 (1.4), OKT3 151 (1.6); PBMC–media 151 (1.3), PHA 509 (1.4), OKT 289 (1.1). Again, no statistically significant ($p < 0.01$) differences existed between CBMC and adult PBMC.

Time course of cellular and supernatant IL-2R production following cellular activation. While these studies of cellular and supernatant IL-2R production on days 5 and 7, respectively, suggested that neonatal mononuclear cells were reasonably competent with regard to the production of IL-2R, we undertook experiments to see if the time course of cellular and supernatant IL-2R generation might be temporally different for CBMC as opposed to adult PBMC. When six freshly isolated CBMC and adult PBMC were examined for cellular IL-2R, no IL-2R could be measured (all samples < 62.5 U/ml). As can be seen in Figure 1, the expression of cellular IL-2R at various time points following activation with PHA was similar in CBMC and adult PBMC. In addition, the generation of supernatant IL-2R at various intervals following activation of these same cells (Fig. 1, *right panel*) was comparable in CBMC and adult PBMC.

Table 1. *Stimulus employed to generate cellular IL-2R**

Donor	Media	PHA	OKT3
CBMC # 1	<62.5	766.8	246.2
CBMC # 2	103.9	8474.7	881.7
CBMC # 3	<62.5	1583.9	601.4
CBMC # 4	<62.5	5030.0	679.0
CBMC # 5	<62.5	2042.2	378.7
CBMC # 6	<62.5	1923.7	818.9
Mean	68.0	2424.6	549.5
(×/÷ SEM)	(1.1)	(1.4)	(1.2)
PBMC # 1	150.2	1928.2	1159.7
PBMC # 2	<62.5	608.2	635.5
PBMC # 3	99.8	3246.5	1129.3
PBMC # 4	<62.5	222.5	492.1
PBMC # 5	<62.5	601.2	544.5
PBMC # 6	70.9	1053.4	538.0
Mean	79.9	901.4	702.3
(×/÷ SEM)	(1.3)	(1.5)	(1.2)
Significance	$p > 0.3$	$p > 0.1$	$p > 0.3$

* Cellular IL-2R expression by CBMC and adult PBMC following activation with various stimuli. CBMC and PBMC were cultured *in vitro* with media, PHA, or OKT3 for 5 days. The cells were washed once in PBS containing 25 mM Tris pH 7.4 (TBS) and solubilized in TBS containing 1% Triton X-100. The amount of IL-2R in the detergent-solubilized cell extracts was determined using an enzyme-linked immunosorbent assay (see "Materials and methods").

Table 2. *Stimulus used to generate supernatant IL-2R**

Donor	Media	PHA	OKT3
CBMC # 1	<62.5	285.9	102.4
CBMC # 2	<62.5	2619.2	432.4
CBMC # 3	<62.5	1010.9	377.9
CBMC # 4	<62.5	3270.6	204.5
CBMC # 5	<62.5	1797.7	206.2
CBMC # 6	<62.5	1317.7	420.3
Mean	<62.5	1342.8	258.3
(×/÷ SEM)		(1.4)	(1.2)
PBMC # 1	154.8	1559.1	1447.2
PBMC # 2	<62.5	1052.0	593.1
PBMC # 3	189.4	3437.0	1410.9
PBMC # 4	<62.5	391.0	655.6
PBMC # 5	85.8	1012.7	654.4
PBMC # 6	78.0	1201.2	592.2
Mean	95.6	1178.6	821.6
(×/÷ SEM)	(1.2)	(1.3)	(1.2)
Significance	$p > 0.05$	$p > 0.5$	$p < 0.01$

* Supernatant IL-2R production by CBMC and adult PBMC. CBMC and adult PBMC were cultured *in vitro* with media, PHA, or OKT3 for 7 days. The supernatants were harvested and the amount of soluble IL-2R in the supernatant was determined using an enzyme-linked immunosorbent assay (see "Materials and methods").

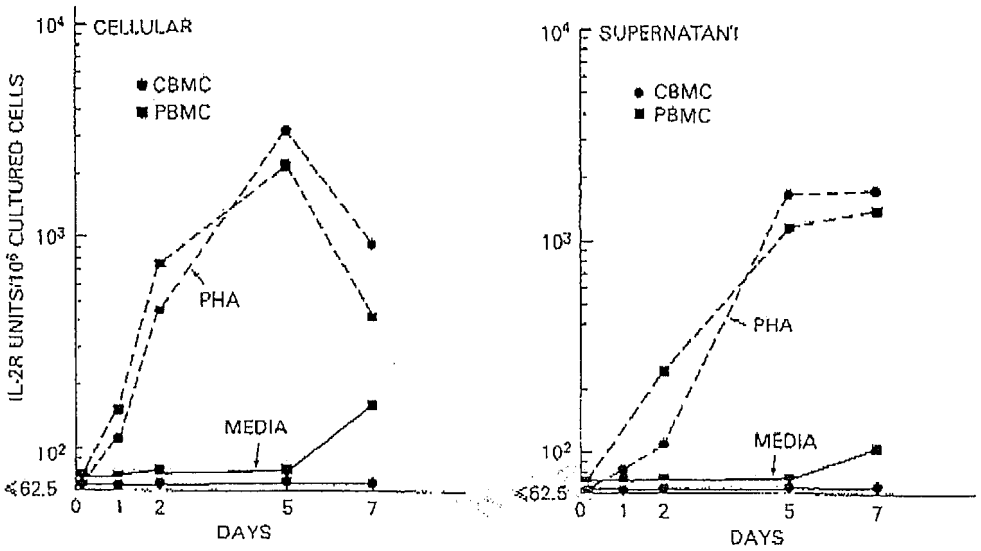

Fig. 1. Time course of cellular and supernatant IL-2R production by PHA-activated CMBC and adult PBMC *in vitro*. CBMC (●) and PBMC (■) were cultured with media alone (——) or PHA (– – –) for various time periods *in vitro*. Detergent-solubilized cells (*left*) and cell-free culture supernatants (*right*) were analyzed for IL-2R by enzyme-linked immunosorbent assay (see "Materials and methods").

Levels of IL-2R in cord blood plasma. As normal adults possess resting serum/plasma levels of IL-2R (Rubin LA, Kurman CC, Fritz ME, Yarchoan R, Nelson DL, unpublished data), we determined the plasma levels of IL-2R in cord blood plasma. The mean (x/÷ SEM) plasma IL-2R level in eight cord blood plasma samples was 499 (x/÷ 1.1) with a range of 335–760, a value not significantly different ($p > 0.1$) than that of 19 normal adults, mean = 548 (x/÷ 1.2), with a range of 155–1640.

DISCUSSION

In the present studies we have examined the capacity of CBMC to generate both cell-associated and released soluble IL-2R *in vitro*. Freshly isolated CBMC like normal adult PBMC did not express measurable IL-2R prior to activation, and cord blood plasma contained levels of soluble IL-2R comparable to adult plasma. Following cellular activation *in vitro* with PHA and the murine monoclonal antibody OKT3 reacting with the T-cell antigen receptor complex, CBMC produced both cellular and supernatant IL-2R. The amounts of IL-2R produced were comparable to those made by adult mononuclear cells, with the exception that CBMC produced significantly less supernatant soluble IL-2R when stimulated with OKT3. No differences were observed between cord blood mononuclear and adult cells in the kinetics of IL-2R production following PHA stimulation.

Recent studies have demonstrated that IL-2 plays a critical role in the maturation and regulation of the immune response (8). Previous studies had demonstrated that IL-2 production by PHA-stimulated cord blood cells is normal (10). The normal proliferative responses observed with CBMC suggested that IL-2R expression by such cells might be normal. Our interest, however, was in whether CBMC might produce increased amounts of cellular and/or soluble IL-2R following activation. As the released *in vitro* soluble IL-2R is capable of binding IL-2 (Rubin LA, Jay G, Nelson DL, unpublished data), these molecules are candidates for mediating immunosuppression, a phenomenon well recognized with CBMC (5–7, 10). In addition, our previous studies using the monoclonal antibody 7G7B6 and flow microfluorimetry suggested that CBMC produced more cellular IL-2R than adult mononuclear cells (16). In the present studies we found that with PHA activation, CBMC produced cellular and supernatant IL-2R levels comparable to adult cells, and with OKT3 stimulation the production of cellular but not supernatant IL-2R was equivalent to adult cells. These differences might be due to the fact that PHA and OKT3 stimulate T cells via different cell surface molecules (17, 18), or alternatively, may relate to the fact that OKT3 stimulates cells via the T-cell antigen receptor—a complex which may not be fully functional in neonatal T cells. It should be stressed, however, that the differences were only observed for supernatant IL-2R and that all CBMC made supernatant IL-2R. In addition, it should be pointed out that the IL-2R present in the culture supernatants from CBMC is known only to be immunoreactive receptor, and studies are planned to attempt to compare the binding affinity of the soluble IL-2R from CBMC and adult PBMC. In soluble form, OKT3 stimulation of T cells is monocyte dependent (19). The present results suggest that this monocyte function is largely normal among neonatal monocytes, a finding consistent with previous work on antigen presentation by such cells (20, 21). The present studies with OKT3 suggest that activation of neonatal T cells through the antigen receptor complex is possible and such activated cells express normal amounts of IL-2R. However, these activated cells may release less IL-2R than adult cells. The mechanism(s) for this hyporesponsiveness remain to be elucidated. Previously we had interpreted the levels of IL-2R in the serum/plasma of normal healthy adults as being the result of ongoing stimulation of the immune system with everyday exposure to exogenous antigens (Rubin LA, unpublished data). The finding of plasma levels of IL-2R in cord blood comparable to adults suggests that this IL-2 might be the result of lymphopoiesis or be of maternal origin rather than exogenous antigenic stimulation. Abnormal levels of plasma or serum levels of IL-2R in pediatric patients might therefore be indicative of immunodeficiency diseases and/or lymphoproliferative disorders.

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